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Fasting and refeeding affect the expression of the Inhibitor of DNA Binding (ID) genes in rainbow trout (*Oncorhynchus mykiss*) muscle

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Abstract

The Inhibitor of DNA Binding/Differentiation (ID) proteins are a family of dominant negative regulators of the basic helix-loop-helix (bHLH) transcription factors, shown in mammals to delay cell differentiation and prolong proliferation. In the current study we used real-time PCR to investigate the effects of fasting and refeeding on the expression of ID genes in rainbow trout muscle. Fry shortly following yolk-sac absorption (~250 mg) were used in a pair of experiments. In the first experiment, the treatment groups included fish fed or fasted throughout the duration of the experiment, and fish fasted for 14 days followed by feeding for the remainder of the experiment. The second experiment consisted of the same treatment groups; however the fish were only fasted for 7 days prior to refeeding. In both experiments, ID gene expression in the muscle of fasted fish was significantly lower than the fed samples after 7 days. Refeeding for 3 or 7 days returned the ID expression to levels similar to the fed fish. The reduction of ID expression during a fast and the subsequent return to fed levels with refeeding suggests the ID proteins participate in the regulation of muscle growth in the rainbow trout.

Keywords: Inhibitor of Differentiation/DNA Binding; ID genes; Fasting; Muscle; Gene expression; Real-time PCR; Rainbow trout; Growth

1. Introduction

During spawning and migration many fish experience an extended period of no or limited food intake. This requires the mobilization of substantial portions of protein reserves stored in the white muscle (Beaulieu and Guderley, 1998; Fauconneau and Paboeuf, 2000; Guderley et al., 2003). The breakdown of muscle proteins is controlled by a number of proteolytic pathways and the expression and activities of these pathways indicate a complex mechanism of regulation (Martin et al., 2002; Salem et al., 2005). Additionally, fasting has been shown to significantly reduce the proliferative potential of myosatellite cells as compared with full fed individuals (Fauconneau and Paboeuf, 2000). However, the mechanisms regulating the gene expression underlying the alteration in the proteolytic expression and the proliferative potential of the satellite cells have yet to be fully understood.

The Inhibition of DNA Binding or Inhibitor of Differentiation (ID) proteins are named for their ability to bind to basic helix-loop-helix (bHLH) transcription factors to attenuate DNA binding and delay cellular differentiation (Benezra et al., 1990). A combination of knockout (Lyden et al., 1999; Mori et al., 2000) and ectopic expression (Atherton et al., 1996; Alani et al., 1999; Wong et al., 2004) experiments in mammals suggest the ID proteins have a role in muscular, neural, immune and mammary tissue development. For example, ID proteins can bind to E-proteins in a muscle cells and prevent the heterodimerization to the myogenic regulatory factors (such as MyoD and myf-5) and thereby delay muscle cell differentiation (Jen et al., 1992).

In the rainbow trout, six ID genes have been characterized (Rescan, 1997; Gahr et al., 2004; Ralliere et al., 2004; Gahr et al., 2005). These genes represent four ID1 transcripts and two ID2 transcripts, based on homologies to mammalian ID proteins, which have likely evolved as a result of a complex combination of gene and genome duplication events (Gahr et al., 2005). Expression of the ID1 and not the ID2 transcripts was

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observed to peak during the mid period of embryonic development (Gahr et al., 2005). Embryonic expression of ID proteins was localized to the somites suggesting a prominent role in muscle development in rainbow trout (Ralliere et al., 2004). Rescan (1997) found that expression of ID1A was restricted to red muscle in the immature fish suggesting a continued role in muscle growth and development beyond embryogenesis.

Muscle growth in the rainbow trout is impacted by genetics, nutritional status, developmental stage and reproductive state (Clawson et al., 1991; Overturf et al., 2003; McKenzie et al., 2003). The mechanisms by which these factors affect muscle growth and development are of interest to the rainbow trout aquaculture industry, as with any meat producing industry. Although developmental and tissue specific patterns of ID protein expression suggest a role for ID proteins in muscle growth and development, the regulation of the ID genes in these processes has not been investigated in fish. Here, we have characterized expression of ID transcripts during a prolonged fast and subsequent refeeding as a first step towards elucidating their roles in regulation of muscle growth and development in fish.

2. Materials and methods

2.1. Experimental design

2.1.1. Fish handling

The experiments were conducted at the National Center for Cool and Cold Water Aquaculture. Rainbow trout were reared in 4 L buckets supplied with flow-through oxygen saturated well water at 12.5±0.2 °C. To maintain water quality, excess feed and wastes were siphoned out of the buckets every other day. Fish were maintained on a 12 h:12 h light/dark regime during the experiments. The trout were fed a commercial diet (55% protein and 15% fat, Zeilger Bros. Inc., Gardner PA) at 2% of body weight. The experimental design and sampling procedures were reviewed and approved by the NCCCWA Institutional Animal Care and Use Committee.

2.1.2. Experiment 1

Immediately following yolk-sac absorption (~250 mg,) fry were separated into 36 buckets containing 33 fish each. Tanks were randomly assigned one of three treatment groups: (1) fed at 2% body weight throughout the experiment (fed, 2 buckets per sample day), (2) not fed throughout the experiment (fasted, 2 buckets per sample day) or (3) not fed for the first 14 days then fed at 2% body weight for the remainder of the experiment (refed, 2 buckets per sample day). Following a 12–16 h fast, fry were euthanized with 200 mg/L Tricaine Methane Sulfonate (Western Chemical, Ferndale WA) before sampling. Muscle samples were collected from two tanks for the fed and fasted treatments prior to initiation of the experiment and on days 3, 7, 14, 17, 21 and 28 of the experiment. The refed group was sampled at initiation of feeding and at 3, 7 and 14 days following refeeding (days 14, 17, 21 and 28 of the experiment). For muscle to be collected, euthanized fish were decapitated, eviscerated and skinned to allow a collection of a clean mixed tissue type muscle sample from each individual. Individual muscle samples were not large enough to enable collection of a suitable quantity of RNA for gene expression analysis. Therefore, six pools containing muscle from four different individuals each were collected for each treatment group and flash frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

2.1.3. Experiment 2

In this second experiment, fry following yolk-sac absorption were separated into 12 tanks containing 20 fish in each tank. Tanks were randomly assigned one of three treatment groups: (1) fed at 2% body weight throughout the experiment (fed), (2) not fed throughout the experiment (fasted); or (3) not fed for the first 7 days then fed at 2% the body weight for the remainder of the experiment (refed). On day 0 samples were collected from 2 fed tanks, on day 7 samples were collected from 2 fed and 2 fasted tanks, and on day 14 samples were collected from 2 fed, 2 fasted and 2 refed tanks. Muscle samples were collected as described above.

2.2. RNA isolation and reverse transcription

Total RNA was isolated from the muscle pools following the TRI-Reagent (Sigma-Aldrich Corp., St. Louis, MO) modification of the guanidine isothiocyanate/phenol–chloroform method (Chomczynski and Sacchi, 1987). The RNA was dissolved in 20–50 μL of nuclease free water and subjected to DNAse treatment following the manufacturer's protocol (DNAse RQ-1, Promega, WI) to remove any genomic DNA contamination. The DNAse treatment was followed with a re-extraction with TRI-Reagent to remove all residual DNAse activity. The quantity of RNA was estimated using a GeneQuant RNA/DNA Calculator (BioChrom Ltd., Cambridge, UK). The quality of the RNA was assessed by agarose gel electrophoresis with the visualization of the 28S and 18S rRNA bands. All RNA samples were stored at −80 °C.

Complementary DNA was synthesized as follows. Two micrograms of total RNA was mixed with 1 μg of random hexamer primers (Promega, Madison, WI) and heated at 70 °C for 5 min. This mixture was cooled on ice before addition of the reverse transcription cocktail containing 1× RT Buffer, 0.5 mM of each dNTP, 25 U of rRNAse Inhibitor (Promega, Madison, WI) and 200 U of MMLV-RT (Promega, Madison, WI) and nuclease free water to a total reaction volume of 40 μL. First strand cDNA synthesis was carried out at 37 °C for 60 min followed by heating the sample to 95 °C for 5 min.

2.3. Real-time PCR and data analyses

All real-time PCR assays were conducted using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using the previously published primers (Gahr et al., 2005). All primers were purchased from Alpha-DNA (Montreal, Canada). For each sample, 1 μl of cDNA was combined with 7.5 μl of 2× SYBR® Green PCR master mix

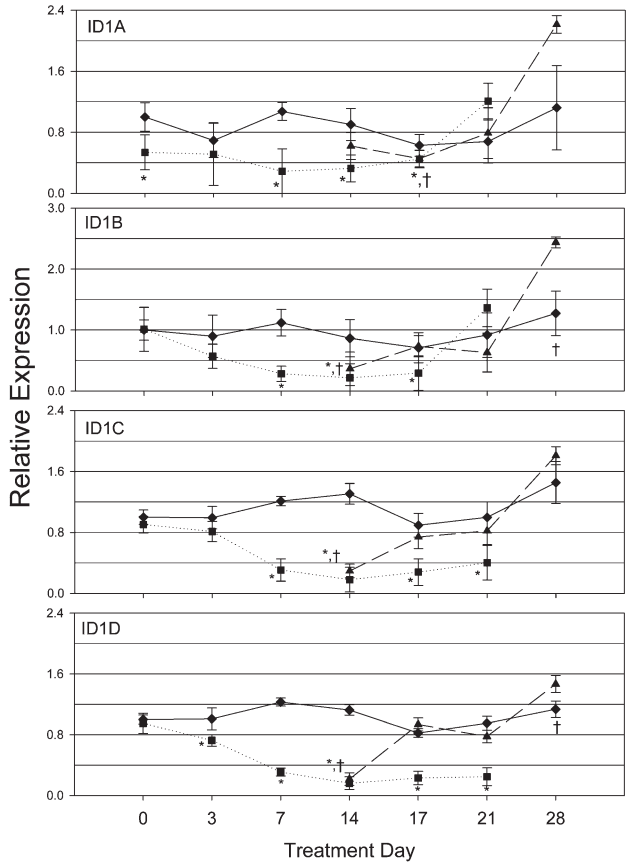

Fig. 1. Expression of the ID1A, ID1B, ID1C and ID1D transcripts for the fed (♦), fasted (■) and refed (▲) groups from experiment 1 shown normalized to β-actin and relative to the samples collected on day 0 (mean±S.E.M., $n=6$ for each sample point). *Significant difference between the fed and fasted groups on each day, †significant difference between the fed and refed groups on each day.

(Applied Biosystems, Foster City, CA). For each reaction, 6 μl of this mixture was added to 9 μl of the primer mix for a 15 μl reaction containing 1X PCR master mix and 500 nM of each primer. The reactions were carried out with the standard reaction conditions as follows, 50 °C for 2 min, 95 °C for 10 min, then 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. The cycling reaction was followed by a dissociation curve to verify amplification of a single product. Correct product amplification was verified by DNA sequencing. To determine the efficiency of the PCR reaction, a serial dilution of three log units was made with a pool of the cDNA from mixed rainbow trout cDNA samples.

The efficiency of the PCR reactions were 1.94, 2.07, 1.92, 1.98, 1.93, 1.91, and 1.88 for ID1A, ID1B, ID1C, ID1D, ID2A, ID2B and β-actin, respectively. Determinations of the relative gene expression levels were made using the $\Delta\Delta C_t$ method as previously described (Gahr et al., 2005). Preliminary analysis revealed the expression of β-actin, Elongation Factor 1α and 18 S rRNA were stable across treatments in the current study. β-Actin was selected for normalization because of its reaction efficiency and relative transcript abundance. The fed samples collected at time zero were used as the control in the calculation of gene expression. Data were analyzed for significant differences using a full factorial model including time, treatment and their interactions (SAS Proc GLM, SAS Institute Inc., Cary, NC) followed by Tukey's Studentized Range (HSD) Test for assignment of significant differences both among treatments for the same day and among time points within a treatment. A p value of 0.05 or less was considered significant.

3. Results

In the first experiment, after just 3 days of fasting, ID1D and ID2B transcript levels in the fasted individuals were significantly lower than the fed fish (Figs. 1 and 2). By the seventh day of fasting, the fasted individuals showed lower transcript levels of all the ID genes and remained lower through day 17 (Figs. 1 and 2). On day 21 of fasting, ID1C and ID1D remained lower in the fasted animals, however, the remainders of the transcripts were at levels similar to the fed samples at this time (Figs. 1 and 2). Due to high mortality in the fasted group between day 21 and day 28, fasted samples could not be collected on day 28. No mortalities were observed in any of the sample tanks before day 21 except for the loss of 4 fish in one of the fasted tanks on day 17. By day 21, one of the fasted tanks had lost 7 and the other 8 animals.

On day 14 of the experiment, fish in the refed group were sampled before feeding and all of the genes except ID1A were lower than the fed group at the same time (Figs. 1 and 2). After just 3 days of refeeding, only ID1A was found to be lower than the fed, and by 7 days all the ID transcripts were at similar levels to the fed (Figs. 1 and 2). Only ID1B, ID1D (increased) and ID2B (decreased) were significantly different from the fed group 14 days after feeding resumed (Figs. 1 and 2).

As a result of the increase in expression of the ID1A and ID1B ($p<0.01$) transcripts in the fasted fish and the reduction in the expression levels of the ID2 ($p<0.01$) transcripts between

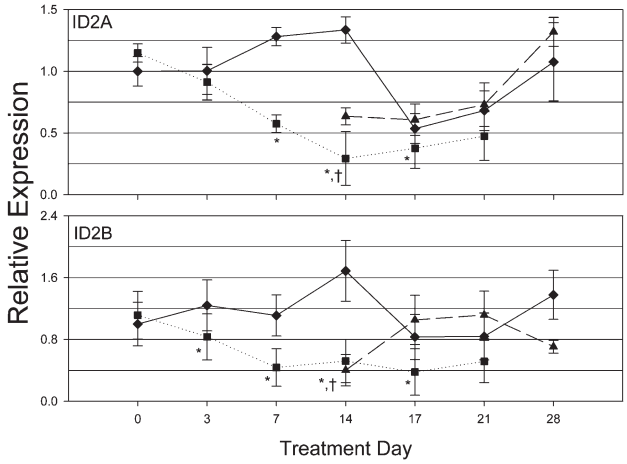

Fig. 2. Expression of the ID2A and ID2B transcripts for the fed (♦), fasted (■) and refed (▲) groups from experiment 1 shown normalized to β-actin and relative to the samples collected on day 0 (mean±S.E.M., $n=6$ for each sample point). *Significant difference between the fed and fasted groups on each day, †significant difference between the fed and refed groups on each day.

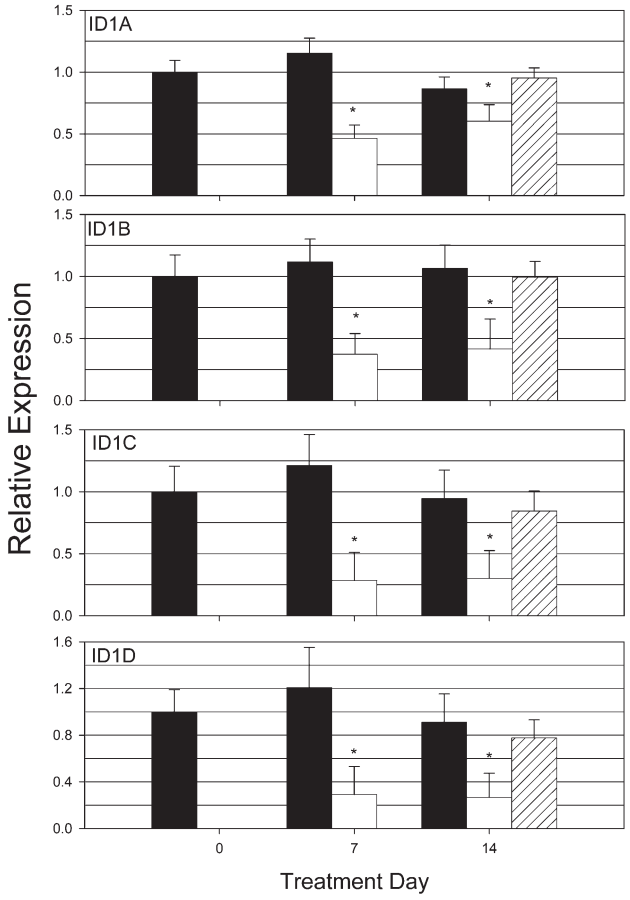

Fig. 3. Expression of the ID1A, ID1B, ID1C and ID1D transcripts for the fed (black bars), fasted (white bars) and refed (cross-hatched bars) groups from experiment 2 shown normalized to β-actin and relative to the samples collected on day 0 (mean±S.E.M., $n=6$ for each sample point). *Significant difference between the fed and fasted groups on each day.

days 17 and 21 in the fed fish, a second experiment was conducted to investigate the effects of refeeding at an earlier time point. In this experiment, we observed that following a 7 day fast all of the ID transcript levels were again significantly lower than for the fed (Figs. 3 and 4). Refeeding for 7 days allowed recovery to similar levels compared to the fed fish for all of the ID1 transcripts. Neither of the ID2 transcripts was significantly different in any of the groups tested on day 14 despite the mean of the refed fish being approximately double that of the fasted fish (Fig. 4). There were significant effects of time for all treatments in experiment 1 ($p>0.01$ for each gene) but no effect in experiment 2. These results might suggest effect of developmental stage in these young animals but the data are not presented or discussed in further detail because the subject is beyond the intended scope of the manuscript. The differences in patterns of expression among the treatments do not suggest they result from a delay in developmental stage in response to fasting.

4. Discussion

Extended periods of fasting (winter, spawning and migration) are a natural part of the life cycle of many fish species and they have the ability to mobilize large quantities of nutrient reserves from muscle with full recovery upon refeeding (Navarro and Gutierrez, 1995). In the current study, we have looked at the effects of prolonged fasting and refeeding on the expression of the ID transcripts in muscle. We observed that by one week of fasting, the expression of all the IDs was significantly reduced in the muscle of fasted animals as compared to the fed animals (Figs. 1–4). To our knowledge, this is the first characterization of ID gene expression during a nutrient deficiency in any vertebrate. However, the utilization of muscle protein during starvation is a form of muscle degradation that is in some ways similar to that found in muscle atrophy or wasting. Alway et al. (2002a) reported a reduced ID expression during hindlimb unloading in the rat, in conjunction with muscle atrophy.

Following refeeding, we found expression of the IDs returned to levels similar to the fed fish (Figs. 1–4) and possibly even higher expression for the ID1s 14 days following the return to feed (only significant for ID1B and ID1D, Fig 1). Brodeur et al. (2002) found that refeeding following a 26 day fast in the Antarctic fish *Notothenia coriiceps* resulted in an increase in the myogenic regulatory factor MyoD and proliferating cell nuclear antigen (PCNA), but no change in the expression of myogenin. This indicates increased recruitment of cells to the myogenic lineage with a concurrent increase in the proliferation of these cells and a reduction in the rate of muscle cell differentiation. In keeping with this interpretation, increased expression of the ID proteins during refeeding period would enhance the delay in differentiation and enhance proliferation of the muscle cells upon refeeding. Additionally, Alway et al. (2002b) found increased expression of ID1 and ID2 genes in the hindlimb of overloaded rat muscles. We are unable to explain the observed reduction in the ID2B expression following the 14-day refeeding in the first experiment. Overall, the increased expression of the IDs following refeeding may suggest a role for the ID proteins in the stimulation of muscle

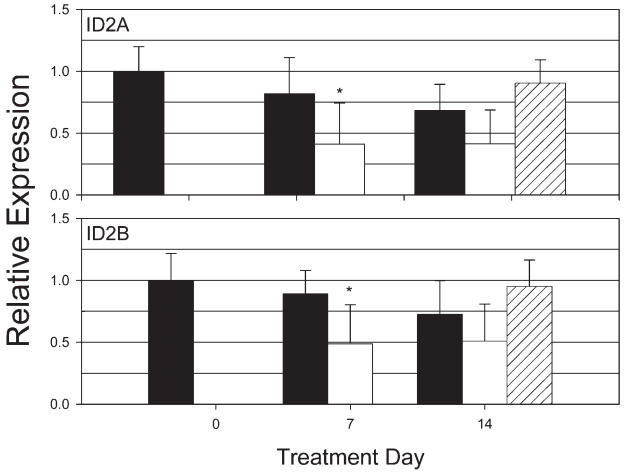

Fig. 4. Expression of the ID2A and ID2B transcripts for the fed (black bars), fasted (white bars) and refed (cross-hatched bars) groups from experiment 2 shown normalized to β-actin and relative to the samples collected on day 0 (mean±S.E.M., $n=6$ for each sample point). *Significant difference between the fed and fasted groups on each day.

cell proliferation during a muscle accretion period, such as refeeding following a prolonged fast.

Fauconneau and Paboeuf (2000) observed delayed in vitro proliferation of muscle satellite cells isolated from fasting as compared to fed rainbow trout. They also observed that 4 days of refeeding resulted in similar proliferation rates of the myosatellite cells between the fed and refed fish. In addition, they also showed stimulation with fetal bovine serum (FBS) enabled the myosatellite cells from fasted fish to eventually reach a similar level of proliferation as cells from fed fish. ID expression in vitro has been found to increase in response to FBS in murine hematopoetic cells (Navarro et al., 2001), suggesting an increase in ID expression in response to FBS may mediate the FBS-induced increase in proliferation of myosatellite cells from the fasted fish. The results of these studies together with our observed changes in ID transcripts in response to fasting and refeeding are consistent with the notion that reduced expression of the IDs during fasting may lower the proliferative potential of the myosatellite cells in the rainbow trout.

The reduced expression of the ID proteins during the fasting period and increase following refeeding could in turn be in response to changes in IGF-I. Previous studies have found a significant decrease in the circulating levels of IGF-I during a fast in other salmonids (Duan and Plisetskaya, 1993, Fukada et al., 2004). Following a prolonged fast, Chauvigne et al. (2003) found expression of IGF-I in the muscle increased with refeeding. In mouse embryo fibroblast cells, Belletti et al. (2002) showed induction of ID1 expression following treatment with IGF-I. Additionally, at least three signaling pathways downstream of the IGF-I Receptor have been shown to increase expression of ID2 in mouse cells (Navarro et al., 2001). Taken together, changes in IGF-I in response to nutritional state may contribute to parallel changes in levels of ID gene expression in muscle.

Increases in the expression of the ID1A, ID1B and ID2A transcripts were observed in the fasted individuals on day 21 of fasting (Figs. 1 and 2). Atherton et al. (1996) observed that in vitro expression of ID3 delayed apoptosis in C_2C_{12} myoblasts. Similarly, Asirvatham et al. (2006) showed increasing ID2 expression in cancer cell lines suppressed apoptosis. Therefore in the current study, the increased expression of ID transcripts following the prolonged fast may have been to provide protection against apoptotic death of the muscle cells. Unfortunately, this increase coincided with increased expression due to the return to feeding. To confirm that the increased expression in the refed group was the result of refeeding and not the preceding fast, we ran the second experiment with a shorter 7 day fast, so that the refeeding would parallel a continued depression in the ID expression in response to the sustained fast. This experiment confirmed that the recovery of ID transcript levels following a fasting period result from a return to feed (Figs. 3 and 4).

In the current study, we have investigated the effects of fasting and refeeding on the expression of the ID genes in the rainbow trout. We observed that fasting resulted in the reduced expression of the ID genes and refeeding resulted in the restoration of expression to levels similar to that of the fed samples. These data show that expression of the ID genes in rainbow trout muscle are responsive to changes in the nutritional status of the organism.

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